Load of Challenge Marek's Disease Virus DNA in Blood as a Criterion for Early Diagnosis of Marek's Disease Tumors

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SUMMARY. Outbreaks of Marek's disease (MD) in vaccinated flocks still occur sporadically and lead to economic losses. Unfortunately, adequate methods to predict MD outbreaks are lacking. In the present study, we have evaluated whether high load of challenge MD virus (MDV) DNA in peripheral blood could aid in the early diagnosis of MD and in monitoring efficacy of vaccines against MD. One experiment was conducted to simulate field conditions by combining various vaccines (turkey herpesvirus [HVT] and HVT + MDV serotype 2 [SB1]) and challenge viruses (GA, Md5, and 648A). Vaccine efficacy among our experimental groups ranged from 13.3% to 94.2%. Each chicken was sampled three times during the length of the experiment (3, 5, and 15 wk postchallenge [wpc]), and gross lesions were evaluated in chickens that died and at termination of the experiment. DNA was extracted from whole blood and buffy coats from each sample, and the load of challenge MDV DNA and HVT DNA were quantified by real-time polymerase chain reaction. Chickens that developed MD by the end of the experiment had higher load of challenge MDV DNA (threshold cycle [Ct] glyceraldehyde-3-phosphate dehydrogenase [GAPDH]/Ct glycoprotein B [gB] ratios of 1.0, 1.04, and 1.05 at 3, 5, and 15 wpc, respectively) than those that did not develop MD (Ct GAPDH/Ct gB ratios of 0.7, 0.69, and 0.46 at 3, 5, and 15 wpc, respectively). However, load of HVT DNA in blood was not correlated with the development of tumors (Ct GAPDH/Ct HVT ratios from 0.04 to 0.10 in both groups). Vaccinated groups with >75% protection had statistically significant less challenge DNA virus (Ct GAPDH/Ct gB ratios of 0.76, 0.70, and 0.45 at 3, 5, and 15 wpc, respectively) than less protected groups (Ct GAPDH/Ct gB ratios of 0.92, 0.97, and 0.85 at 3, 5, and 15 wpc, respectively). No differences in the load of HVT DNA could be found between protected and nonprotected groups at any time point of the study (Ct GAPDH/Ct HVT from 0.05 to 0.09 in both groups). Our results showed that load of challenge MDV DNA but not load of HVT DNA in blood can be used as criterion for early diagnosis of MD.

RESUMEN. Cantidad de ADN en la sangre del virus de desafío de la enfermedad de Marek como criterio para el diagnóstico temprano de tumores causado por el virus de Marek.

Los brotes de la enfermedad de Marek en lotes vacunados aún ocurren esporádicamente y generan pérdidas económicas importantes. Desafortunadamente, existe una carencia de métodos diagnósticos adecuados para predecir los brotes de esta enfermedad. En el presente estudio evaluamos si una alta cantidad del ADN de un virus de desafío de la enfermedad de Marek en la sangre periférica podría ayudar en el diagnóstico temprano de esta enfermedad y en el seguimiento de la efectividad de las vacunas contra la enfermedad de Marek. Se llevó a cabo un experimento para simular las condiciones de campo combinando varias vacunas: virus Herpes de pavo (HVT), HVT + virus de la enfermedad de Marek serotipo 2 (cepa SB1), y varios virus de desafío (cepas GA, Md5 y 648A). La efectividad de la vacuna entre los grupos experimentales estuvo en un rango desde 13.3% a 94.2%. A cada ave se le tomaron muestras tres veces a lo largo de todo el período experimental (3, 5 y 15 semanas después del desafío) y las lesiones macroscópicas fueron evaluadas en las aves que morían y al finalizar el experimento. El ADN fue extraído de sangre completa y de linfocitos periféricos de cada muestra y la cantidad de ADN del virus de desafío y el ADN de la vacuna HVT fueron cuantificadas mediante la prueba de reacción en cadena por la polimerasa en tiempo real. Las aves que desarrollaron la enfermedad de Marek al final del experimento tenían cantidades más altas del ADN del virus de desafío de la enfermedad de Marek [proporción ciclo – umbral (Ct, por su sigla en Inglés) gliceraldehído-3-fosfato deshidrogenasa (GAPDH, por su sigla en Inglés)/Ct glicoproteína B (gB, por su sigla en Inglés) de 1.0, 1.04 y 1.05 a 3, 5 y 15 semanas después del desafío, respectivamente] que aquellos que no desarrollaron la enfermedad (proporción Ct GAPDH/Ct gB de 0.7, 0.69, y 0.46 a 3, 5 y 15 semanas después del desafío, respectivamente). Sin embargo, la cantidad del ADN de la vacuna HVT no estuvo correlacionada con el desarrollo de tumores (proporción Ct GAPDH/Ct HVT de 0.04 a 0.10 en ambos grupos). Los grupos vacunados con >75% de protección tuvieron estadísticamente significante menor cantidad de ADN de virus de desafío (proporción Ct GAPDH/Ct gB de 0.76, 0.70 y 0.45 a 3, 5 y 15 semanas postdesafío, respectivamente) que los grupos menos protegidos (proporción Ct GAPDH/C de 0.92, 0.97 y 0.85 a 3, 5 y 15 semanas postdesafío, respectivamente). No se encontraron diferencias significativas en la cantidad del ADN del virus vacunal HVT entre los grupos protegidos y no protegidos en ningún momento del estudio (Proporción Ct GAPDH/Ct HVT desde 0.05 a 0.09 en ambos grupos). Nuestros resultados mostraron que la cantidad de ADN del virus de desafío de la enfermedad de Marek, pero no la cantidad de ADN de la vacuna HVT en la sangre, puede ser usada como criterio para el diagnóstico temprano de la enfermedad de Marek.

Key words: Marek's disease, vaccination, control, protection, monitoring

Abbreviations: bp = base pair(s); CEF = chicken embryo fibroblast; Ct = threshold cycle; DEF = duck embryo fibroblast; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; gB = glycoprotein B; HVT = turkey herpesvirus; MD = Marek's disease; MDV = Marek's disease virus; np = nonprotected; ORF = open reading frame; p = protected; PCR = polymerase chain reaction; PFU = plaque-forming units; SB1 = MDV serotype 2; wpc = weeks postchallenge

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Marek's disease (MD) is a lymphoproliferative disease of chickens that is of great concern for the poultry industry. Even though MD has been successfully controlled by vaccination since 1968 (5), sporadic outbreaks of MD still occur, and they lead to remarkable economic losses. There are many different factors that contribute to the onset of an outbreak. The evolution of Marek's disease virus (MDV) toward more virulence has a negative effect on the vaccine efficacy (16,20). In addition, MD vaccines are cell-associated; therefore, they are very labile. Problems associated with storing and handling of MD vaccines are responsible for many of the outbreaks (13). Concomitant immunosuppressive diseases (i.e., chicken infectious anemia, infectious bursal disease) can also decrease the efficacy of MD vaccines and contribute to their failure (11). Finally, other factors such as early exposure to MDV before development of immunity against the disease also play a role.

Monitoring of MD protection in the field is extremely difficult because MDV is ubiquitous and infection is not synonymous with disease. Moreover, even though there is strong neutralizing antibody response after MD vaccination, neutralizing antibodies do not protect against development of tumors (14). Therefore, they cannot be used to estimate the level of protection conferred by MD vaccine.

Several attempts have been made to develop methods to monitor the efficacy of MD vaccines in the field. Okazaki and coworkers in 1973 (12) and Cho and coworkers in 1976 (4) suggested an association between turkey herpesvirus (HVT) viremia and protection against MD development. Recently, Baigent and coworkers developed a real-time polymerase chain reaction (PCR) assay to measure MDV DNA load in feather pulp (1,2). They proposed this method to evaluate proper vaccine administration, and they suggested that it might also be valid to monitor protection against MD (3).

We have recently shown that amount of MDV DNA present in MD-induced tumors is a very useful criterion to diagnose MD (7). Islam and coworkers have shown that there is more MDV in buffy coats of chickens with MD tumors (10). The aim of this study is to develop a method of early MD diagnosis that can predict efficacy of MD vaccination. In particular, the objectives of this work were 1) to determine whether the load of challenge MDV DNA in peripheral blood and/or buffy coats at different time points correlates with development of tumors and whether this can be used to monitor vaccine-induced protection in our experimental model; and 2) to determine whether the load of HVT DNA in peripheral blood and/or buffy coats at different time points correlates with development of tumors and whether this can be used to monitor vaccine-induced protection in our experimental model.

MATERIALS AND METHODS

Chickens. Chickens were MD-susceptible F1 progeny (15x7) from Avian Disease and Oncology Laboratory line 15I5 males and line 71 females. All breeder chickens were free of antibodies to avian leukosis virus, reticuloendotheliosis virus, and various other poultry pathogens. However, the breeder females had been vaccinated with all three serotypes of MDV to ensure the presence of maternal antibodies in progeny chicks.

Viruses. Oncogenic serotype 1 MDVs used in this study were virulent strain GA at passage 19 in duck embryo fibroblast (DEF) (6), very virulent strain Md5 at passage 8 in DEF (20), and very virulent plus strain 648A at passage 10 in DEF (16). Vaccine strains used were FC-126 at passage 10 in chicken embryo fibroblast (CEF) (18) and MDV serotype 2 (SB1) at passage13 in CEF (15).

Real-Time PCR. Real-time PCR assay was performed as described previously (7). Briefly, DNA was extracted from whole blood and buffy

coats using PuregeneTM DNA Isolation kit (Gentra Systems, Inc., Minneapolis, MN), and each sample was amplified with three pair of primers specific for the glycoprotein B (gB) gene of MDV, for the chicken glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene, and for a 62-bp fragment that lies between open reading frames (ORFs) HVT072 and HVT073 of the HVT genome. The sequence for the respective forward and reverse primers were TM.5 (5′-CGGTGGCTTTTCTAGGTTCG-3′) and TM.3 (5′-CCAGT-GGGGTTCAACCGTGA-3′) for serotype 1 gB gene that amplified a fragment of 66 bp; GAPDH-TM.5 (5′-GGAGTCAACG-GATTTGGCC-3′) and GAPDH-TM.3 (5′-TTTGCCAGAGAG-GACGGC-3′) for chicken GAPDH gene that amplify a fragment of 63 bp; and HVT TM-F2 (5′-CGGGCCTTACGTTTCACCT-3′) and HVT TM-R2 (5′-GCGCCGAAAAGCTAGAAAAG-3′) for a 62-bp fragment that lies between ORFs HVT072 and HVT073 of HVT genome. Amplifications were done using an Mx3005 (Stratagene, La Jolla, CA) in a 25-µl PCR reaction containing 50 ng of DNA, 0.2 µM each primer, and SYBR® Green PCR master mix (Brilliant® SYBR® Green) that contains the appropriate buffers, nucleotides, and Taq polymerase (Biocrest-Stratagene, Cedar Creek, TX). The reaction was cycled 50 times at 95 C denaturation for 15 sec and a 60 C combined annealing/extension for 60 sec. Fluorescence was acquired at the end of the annealing/extension phase. The melting curves were obtained at the end of amplification by cooling the sample at 20 C/sec to 60 C and then increasing the temperature to 95 C at 0.1 C/sec. The parameter threshold cycle (Ct) was calculated for each PCR reaction by establishing a fixed threshold. Ct is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. Relative quantification of the amount of target in unknown samples was accomplished by the comparative Ct method. Two Ct ratios were established for each sample (Ct ratio GAPDH-gB = Ct GAPDH/Ct gB and Ct ratio GAPDH-HVT = Ct GAPDH/Ct HVT). The higher the Ct ratio, the higher the load of MDV or HVT, respectively.

Experimental design. One experiment was conducted using a combination of vaccines and oncogenic MDV (HVT/GA, HVT/Md5, HVT/648A, HVT + SB1/Md5, and HVT + SB1/648A) that provide different level of protection based on previous experiments (7,16,20). Vaccines were administered subcutaneously at hatch at a dose of 2000 plaque-forming units (PFU). Chickens were challenged with oncogenic virus at 5 days postvaccination (6 days of age), by the subcutaneous route, at a dose of 500 PFU. Samples of blood were collected at 3, 5, and 15 wk postchallenge (wpc), and load of challenge MDV DNA and HVT DNA was evaluated from whole blood and buffy coats. Details of the experimental groups and virus strains used are shown in Table 1.

Statistical analysis. Data were analyzed at individual level to study correlation between load of viral DNA (challenge MDV and HVT) and development of tumors in individual chickens and at group level to study correlation between load of viral DNA (challenge MDV and HVT) and protection of vaccinated groups. For the individual study, chickens were divided into two categories (lesions and no lesions) based on the presence or absence of MD lesions at death of the chicken or at termination of the study (regardless of the treatment group). For the group analysis, treatment groups were divided into two categories (protected [p] and nonprotected [np]) based on an arbitrary criterion: <25% of the chickens developed lesions in p groups; >25% of the chickens developed lesions in the np groups. For the group analysis, all chickens from a given treatment group were included in the study regardless of the presence or absence of lesions. The statistical program STATISTICA (StatSoft, Tulsa, OK) was used. Comparisons among groups were conducted by a Student *t*-test. The level of significance considered was $P < 0.05$.

RESULTS

Validation of experimental model. To achieve different levels of protection conferred by vaccination, we have used the challenge model developed by Witter (16) in the pathotyping studies. Table 1

Table 1.   Validation of the challenge model.

Lot	No. chickens	Level of protection[A]	Vaccine			Challenge			Expected MD%[B]	Actual MD%[C]
			Strain	Dose (PFU)	Age (days)	Strain	Dose (PFU)	Age (days)		
1	17	p	HVT	2000	1	GA	500	6	<25	5.8
2	17	np	HVT	2000	1	Md5	500	6	>25	76.5
3	17	np	HVT	2000	1	648A	500	6	>25	86.7
4	17	p	HVT + SB1	1000 + 1000	1	Md5	500	6	<25	18.7
5	17	np	HVT + SB1	1000 + 1000	1	648A	500	6	>25	53.3
6	17	NA[D]	—	—	—	—	—	—	0	0.0

[A]Level of protection is based on the expected percentage of MD when using a particular combination of vaccine and challenge virus. An arbitrary value of 25% was considered as a cut-off point: p <25% and np >25%.
[B]Percentage of expected tumors are based on the results of previous studies (7,16,20).
[C]Actual percentage of MD indicates the results obtained in this study.
[D]NA = not applicable.

shows that percentage of chickens developing MD tumors in the present study did not differ from results obtained in previous work (16); therefore, the experimental model used in this study was validated. Less than 25% of the chickens were affected in groups vaccinated with HVT and challenged with GA (5.8%) and vaccinated with HVT + SB1 and challenged with Md5 (18.7%). These two groups were considered as p groups. In contrast, >25% of the chickens vaccinated with HVT and challenged with Md5 (76.5%), vaccinated with HVT and challenged with 648A (86.7%), and vaccinated with HVT + SB1 and challenged with 648A (53.3%) developed tumors. These three groups were considered as non-protected.

Individual analysis: correlation between load of viral DNA and development of tumors. *Correlation between load of challenge MDV DNA and development of tumors.* Chickens that developed tumors had higher load of MDV in both peripheral blood (ratios 1.00, 1.04, and 1.05 at 3, 5, and 15 wpc, respectively) (Fig. 1A) and buffy coats (ratios 1.02, 1.00, and 1.02 at 3, 5, and 15 wpc, respectively) (Fig. 1B) than chickens that did not develop tumors (ratios 0.70, 0.69, and 0.46 in whole blood and 0.82, 0.55, and 0.55 in buffy coats at 3, 5, and 15 wpc, respectively). Differences were statistically significant at 3, 5, and 15 wpc in both whole blood and buffy coats. In each treatment groups, chickens showing MD lesions had higher load of MDV DNA in peripheral blood and in buffy coats than chicken without MD lesions (data not shown).

Correlation between load of HVT DNA and development of tumors. No statistically significant differences in the load of HVT DNA in whole blood (Fig. 1C) or in buffy coats (Fig. 1D) were found between chickens that developed MD lesions (ratios 0.04, 0.09, and 0.10 in whole blood and 0.31, 0.25, and 0.20 in buffy coats at 3, 5, and 15 wpc, respectively) and those that did not develop tumors (ratios of 0.10, 0.04, and 0.04 in whole blood and 0.35, 0.20, and 0.15 in buffy coats at 3, 5, and 15 wpc). Load of HVT DNA was higher when measured in buffy coats than in whole blood, but no differences between chickens with tumors and chickens without tumors could be detected in whole blood or buffy coats any time point. No differences were observed between chickens with MD lesions and chickens without MD lesions within the same treatment group (data not shown).

Group analysis. *Correlation between load of viral DNA and protection.* Correlation between load of challenge MDV DNA and protection: MDV DNA load in peripheral blood (Fig. 2A) and buffy coats (Fig. 2B) of chickens belonging to the better protected groups (HVT/GA, HVT + SB-1/Md5) was significantly lower at 3, 5, and 15 wpc (ratios of 0.75, 0.70, and 0.45 in whole blood and 0.84, 0.49, and 0.64 in buffy coats) than those belonging to the less protected groups (HVT + SB1/648A, HVT/Md5, and HVT/648A)

(ratios of 0.92, 0.97, and 0.85 in whole blood and 0.99, 0.95, and 0.80 in buffy coats).

Correlation between load of HVT DNA and protection. No statistically significant differences in the load of HVT DNA in whole blood (Fig. 2C) or in buffy coats (Fig. 2D) were found between chickens belonging to p groups (HVT/GA, HVT + SB-1/Md5) (ratios of 0.05, 0.048, and 0.05 in whole blood and 0.32, 0.20, and 0.17 in buffy coats at 3, 5, and 15 wpc, respectively) and those belonging to np groups (HVT + SB1/648A, HVT/Md5, and HVT/648A) (ratios of 0.08, 0.09, and 0.05 in whole blood and 0.33, 0.24, and 0.17 in buffy coats at 3, 5, and 15 wpc, respectively). Load of HVT DNA was higher when measured in buffy coats than in whole blood, but no differences between chickens from p groups and chickens from np groups could be detected in whole blood or buffy coats at any time point.

DISCUSSION

In this work, we have used a model to simulate field conditions, in which chickens with different levels of vaccine protection are exposed to MDVs of different virulence. Using this model, we have shown that chickens that develop tumors have higher load of challenge MDV DNA in both whole blood and buffy coats samples, and the differences can be detected as early as 3 wpc. This finding supports the results of Islam and coworkers that reported higher MDV DNA in buffy coats of chickens that develop tumors (10). In addition, our results showed that load of MDV DNA in blood can be use to predict the development of MD in a flock several weeks before the clinical appearance of the disease. Our results showed no major differences between load of challenge MDV DNA measured in whole blood and in buffy coats. Due to the technical advantages of using whole blood *vs.* using buffy coats, authors recommend whole blood to measure load of challenge MDV DNA. The use of other less invasive types of samples (i.e., feather pulp) was not tested in this study, but it might be an alternative to consider in future work.

It has been proposed that detection of vaccine DNA in the feather pulp is an adequate method to monitor vaccines in the field (3). Our result showed that vaccine DNA load in either whole blood or buffy coats is not an adequate method to monitor efficacy of vaccination. Based on our results, load of vaccine DNA in blood indicated that chickens have been properly vaccinated, but it was not related to development of MD tumors. The main difference between previous work and our study is the challenge model used. In previous studies, lack of efficacy of vaccination was based on lower doses of the administered vaccine. In the challenge model used by Baigent *et al.*
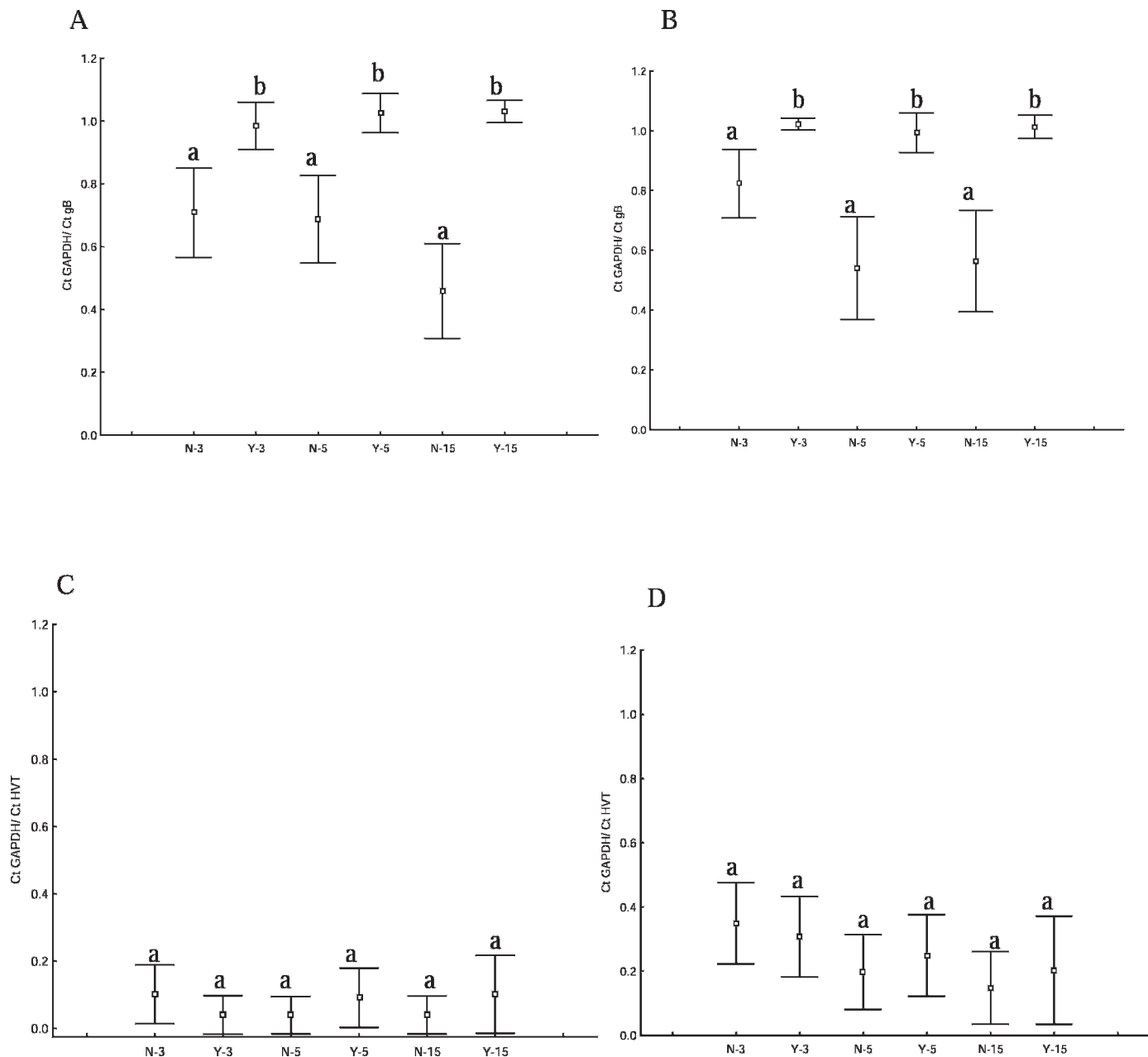
Fig. 1. Load of challenge MDV DNA (A, B) and HVT (C, D) in whole blood (A, C) and buffy coats (B, D). Chickens from all treatment groups were divided into two groups based on the presence (Y) or absence (N) of gross lesions at the end of the experiment (15 wpc). From each chicken, blood was collected at 3, 5, and 15 wpc, and load of viral DNA (challenge MDV and HVT) was measured by real-time PCR in whole blood and in buffy coats. Data are presented as the mean and the 0.95 confidence interval. Differences between chickens without lesions (N) and with lesions (Y) at each time point (3, 5, and 15 wpc) were statistically studied (Student t-test, $P < 0.005$). Within each time point, the same letter placed above the bars means that the differences were not significant.

(3) chickens were vaccinated with CVI988 and challenged 14 days later with a very virulent MDV, RB1B. In that challenge model, if administered properly, CVI988 had the potential of protecting >90% of the chickens. In the challenge model used in this study, the lack of protection is based on the inability of the vaccines to protect against a particular more virulent strain of MDV, even at an adequate vaccine dose.

The presence of the same load of HVT DNA in blood of chickens regardless of the level of protection achieved indicated that break of HVT-induced immunity by more virulent MDV strains is not related to a reduction of HVT ability to replicate *in vivo*. The evolution of MDV toward more virulence has been accompanied by

the reduction of MD vaccine efficacy. The factors associated with the higher ability of more virulent virus strains to break vaccine-induced immunity are not elucidated yet. Our results indicated that virulence of the challenge MDV did not affect the ability of the vaccine HVT to replicate. It has been proposed that strong replication of MDV vaccine is necessary to acquire adequate protection (3,8,17). Results in this study suggest that adequate replication of HVT is not enough to warrant the protection against challenge with very virulent or very virulent plus challenge. However, detection of HVT in blood might not be an accurate way to measure HVT replication. Holland *et al*. (9) demonstrated by *in situ* hybridization that the main site of HVT latency is not the peripheral blood but the spleen. Therefore, it is
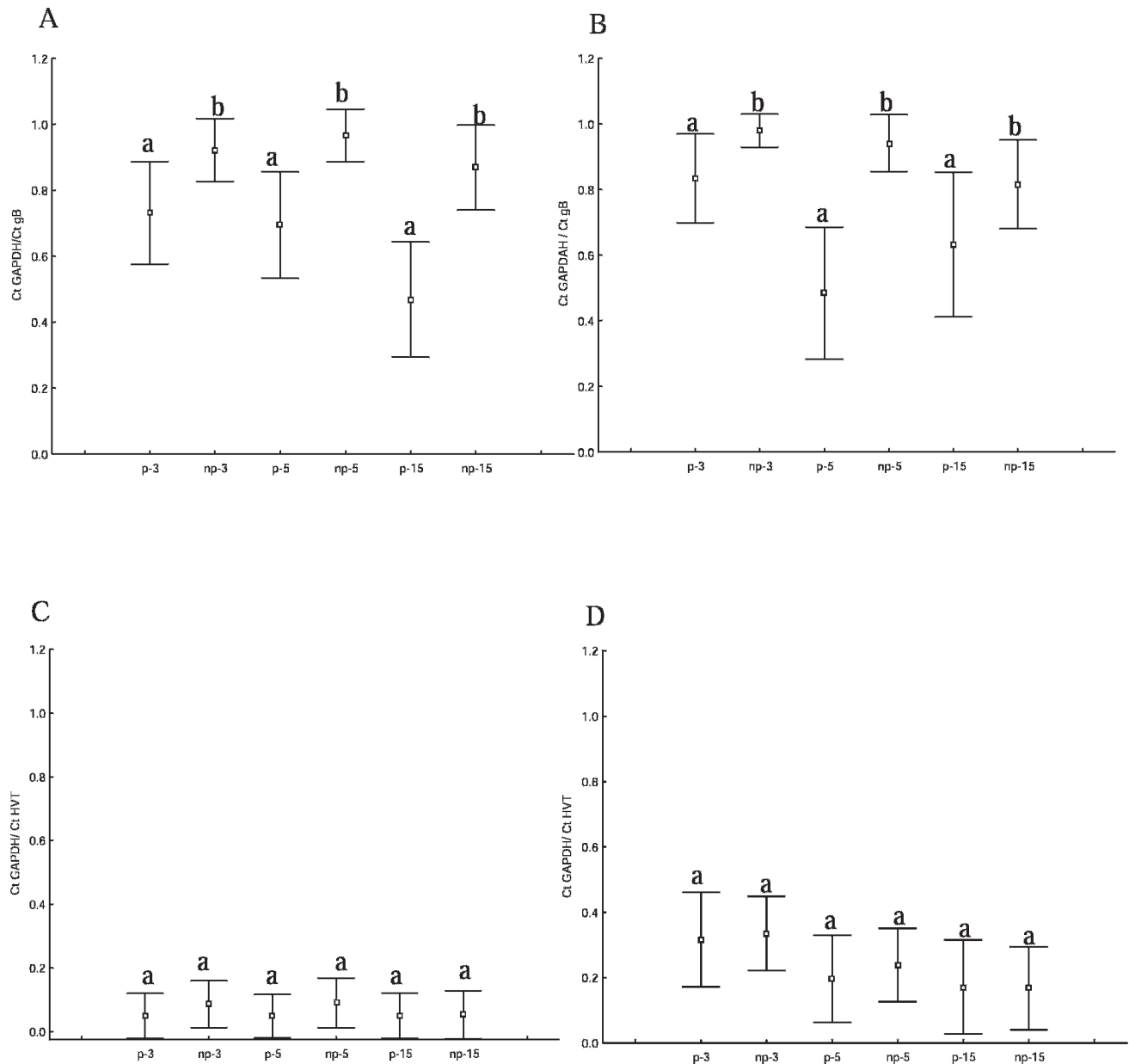
Fig. 2.   Load of wild-type MDV DNA (A, B) and HVT (C, D) in whole blood (A, C) and buffy coats (B, D). Treatment groups were divided into two groups based on the protection conferred by the vaccines. The p groups include chickens vaccinated with HVT and challenged with GA and chickens vaccinated with HVT + SB1 and challenged with Md5. Vaccination in these two groups protected at least 75% of the chickens. The np groups included chickens vaccinated with HVT and challenged with either Md5 or 648A, and chickens vaccinated with HVT + SB1 and challenged with 648A. Vaccination in these three groups protected <25% of the chickens. Samples of blood were collected at 3, 5, and 15 wpc from chickens of each group. Load of viral DNA (challenge MDV and HVT) was measured by real-time PCR in whole blood and in buffy coats. Data are presented as the mean and the 0.95 confidence interval. Differences between chickens without lesions (N) and with lesions (Y) at each time point (3, 5, and 15 wpc) were statistically studied (Student t-test, $P < 0.05$). Within each time point, the same letter placed above the bars means that the differences were not significant.

possible that differences in the load of HVT in spleen might have better correlation with protection.

Challenge MDV DNA load in blood of chickens that develop tumors was higher than in those that did not develop tumors. This finding might be due to the presence of neoplastic cells in the blood of chickens that develop tumors. In a previous study, we have demonstrated that load of MDV DNA in MD tumors is much higher than in latently infected tissues (7). When a vaccine is not conferring adequate protection against a given challenge MDV, chickens develop tumors in various tissues. In this study, 3 wpc was

selected as the first time of sampling because microscopic neoplastic lesions can be detected by that time in chickens experimentally infected with MDV, although clinical signs and gross lesions are still very rare (19). The presence of neoplastic lymphocytes in peripheral blood could be responsible for the higher MDV DNA load in the blood of chickens that will later develop gross tumors (7).

Early detection of chickens that will develop tumors is extremely relevant not only in research but also in the field to monitor the efficacy of vaccination. With this purpose, we tested whether measuring challenge MDV DNA load in blood will be valid to

differentiate groups of chickens well protected by MD vaccines *vs.* groups that were not well protected. Our results showed that statistically significant differences could be detected as early as 3 wpc. These results give some optimism in the development of methods to monitor protection in the field. However, our current challenge model has various limitations for practical use in the field. In the challenge model of this study, we have used only vaccines of serotypes 2 and 3. Those vaccines can be easily differentiated from field type virus by real-time PCR. However, the primers used in this study to detect serotype 1 MDV do not detect differences between serotype 1 MDV vaccines (i.e., CVI988) and challenge MDV. To minimize differences with field conditions, we have used chickens with maternal antibodies against all serotypes of MDV. However, the chicken strain used, 15x7, is very susceptible to MD and differences with the commercial genetic lines might occur. Finally, it is expected that chicks get exposed to MDV in the field at very early age. However, the exact moment when infection occurs is unknown.

Our results show that measurement of challenge MDV DNA but not HVT DNA in blood is an adequate criterion for an early diagnosis of MD under experimental conditions. The model used in this study has the potential of being valid in field conditions when flocks are vaccinated with serotypes 2 and 3 but not with serotype 1 because the primers used in the study will not be able to differentiate between serotype 1 MDVs. Further studies to validate this method in field trials and to optimize methods that permit differentiation between serotype 1 MDVs are warranted.

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